ABSTRACT

Monoclonal antibodies (MAbs) COL-4 and COL-12, to the carcinoembryonic antigen (CEA), and B72.3, CC-49, CC-83, to the tumor-associated glycoprotein 72 (TAG-72), were used to study the expression of distinct epitopes of the two molecules in 71 cases of lung carcinoma of differing histotype. These MAbs reacted with the majority of adenocarcinomas by immunoperoxidase on tissue sections, but demonstrated a more restricted reactivity with squamous carcinomas. MAb CC-49 detected the highest percentages of adenocarcinomas while the B72.3 epitope was expressed more in squamous carcinoma cells. No significant reactivity with any of these MAbs was observed in small cell carcinomas. The expression of the CEA and TAG-72 epitopes in non-small cell lung cancers was highly heterogeneous: a distinct epitope could be expressed by the majority of cells, whereas another of the same antigenic molecule was either poorly or not expressed. In adenocarcinomas, mixtures of anti-CEA, anti-TAG-72, and anti-(TAG-72 plus CEA) MAbs resulted in additive reactivity with an increase of the immunopositive tumors and of the percentages of immunostained cells. This was particularly evident for the anti-TAG-72 plus CEA) mixture. In squamous cell carcinomas the increase was modest and was mainly related to anti-TAG-72 reactivity. These studies suggest variability in the antigenic structure of tumor-associated antigens expressed by carcinomas and indicate that anti-(TAG-72 plus CEA) mixtures may represent an immunoprophylactic advantage for clinical application in adenocarcinoma patients. On the other hand, TAG-72 should be considered a better target antigen, as compared to CEA, in the detection of squamous cell carcinomas.

INTRODUCTION

The heterogeneous expression of tumor-associated antigens in different cancer cells within a given tumor mass as well as in different tumors of a given histological type represents a major caveat to potential applications of monoclonal antibodies in the diagnosis and management of carcinoma patients. In addition, TAAs may be expressed in specific types of normal cells and may be shared by tumors which widen in histological type and clinical behavior (1–7). The high degree of antigenic heterogeneity of carcinomas suggests that panels of MAbs reactive with distinct TAAs and/or with distict epitopes of the same TAA are essential for clinical applications (4, 7).

The carcinoembryonic antigen and the tumor-associated glycoprotein TAG-72 are distinct high-molecular-weight glycoproteins expressed in gastrointestinal, ovarian, mammary, and pulmonary carcinomas (8–18). It was previously shown in gastric carcinomas that the combined use of an anti-TAG-72 MAb (B72.3) and of an anti-CEA MAb (COL-4) allowed the immunological identification of more carcinoma cells within a given tumor as compared to the number of tumor cells reactive with each individual MAb (19). The available data therefore suggest that CEA and TAG-72 represent ideal targets for the study of complementations of anti-TAA MAbs in reactivity to carcinoma. This is greatly facilitated by the availability of several well characterized MAbs, which have been shown to identify distinct epitopes of the TAG-72 and CEA molecules (20–23). In particular, MAbs COL-4 and COL-12 can be distinguished on the basis of their differential reactivities (20) and may recognize biochemically characterized epitopes of the M, 180,000 CEA molecule (21). The anti-TAG-72 MAbs B72.3, CC-49, and CC-83 were extensively characterized by using direct binding RIA, liquid competition RIA, and K, measurements (11, 12, 14, 22, 23). These MAbs could be distinguished from each other on the basis of competition assays and of differential binding in solid-phase RIA.

This study analyzes the immunohistochemical reactivities of these MAbs with 71 cases of lung carcinomas, (a) to define the pattern of expression of the specific epitopes in different histological types of tumor (24), and (b) to determine whether the combined use of these MAbs could increase the number of detectable antigen-positive lung cancers and/or the percentages of immuno reactive tumor cells within each positive tumor.

MATERIALS AND METHODS

Monoclonal Antibodies. MAbs COL-4 and COL-12 were prepared by using extracts or membrane-enriched fractions of biopsy material from primary and metastatic colon carcinomas as sequential immunogens (20). These MAbs were shown by Western blotting to specifically react with the M, 180,000 CEA glycoprotein expressed in neoplastic cells and not with CEA cross-reacting antigens (20). MAb B72.3 was generated against a membrane-enriched fraction of breast carcinoma metastasis and recognizes a high-molecular-weight glycoprotein, designated TAG-72 (10–14). TAG-72 is strongly expressed in a variety of human carcinomas, particularly from the gastrointestinal tract (13–16). MAbs CC-49 and CC-83 were generated against the TAG-72 glycoprotein purified from tumor xenografts of the human LS-174T colon carcinoma cell line (22). All these anti-TAA MAbs were obtained from Dr. J. Schiom, Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, Bethesda, MD.

Tissues and Immunoperoxidase Technique. Paraffin-embedded tissues were selected, after pathological review, from the files of the Departments of Surgery and Anatomic Pathology, University G. D’Annunzio, Chieti, Italy. All tissues were fixed for 6–8 h in 10% buffered formalin. The sample studied included 25 adenocarcinomas, 36 squamous cell carcinomas, and 10 small cell carcinomas. Paraffin sections cut at 5 μm from blocks representative of the tumor and of normal pulmonary tissue were routinely utilized for immunoperoxidase staining. Immunohistochemical reactions were always compared in serial sections rigidly controlled in thickness. Frozen samples from 5 squamous cell carcinomas and 5 adenocarcinomas were also used for the preparation of cryostat sections differentially fixed in cold acetone, Cytofix (Shandon), 5% buffered paraformaldehyde, or were air dried. Paraffin sections were dewaxed in xylene, hydrated through graded ethanol, and washed in 0.01 M PBS, pH 7.4. Endogenous peroxidase activities were blocked by immersion in 0.3% (v/v) hydrogen peroxide in absolute methanol for 30 min. For immunoperoxidase staining we used a streptavidin-biotin system kit for primary mouse antibodies (Zymed, San Francisco, CA).
Fig. 1. Patterns of immunostaining (arrows) observed in normal bronchial epithelium with anti-CEA MAbs (A, COL-4) and with anti-TAG-72 MAbs (B, CC-49). Immunoperoxidase, counterstained with hematoxylin; × 250.

Table 1 Immunoperoxidase staining of lung carcinomas with the anti-CEA MAbs (COL-4 and COL-12) and anti-TAG-72 MAbs (B72.3, CC-49, and CC-83).

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>No. of positive/no. of cases</th>
<th>COL-4</th>
<th>COL-12</th>
<th>B72.3</th>
<th>CC-49</th>
<th>CC-83</th>
</tr>
</thead>
<tbody>
<tr>
<td>(88)%</td>
<td></td>
<td>(92)%</td>
<td>(96)%</td>
<td>(96)%</td>
<td>(84)%</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinomas (36)</td>
<td></td>
<td>27/36</td>
<td>18/36</td>
<td>29/36</td>
<td>29/36</td>
<td>22/36</td>
</tr>
<tr>
<td>(75)%</td>
<td></td>
<td>(50)%</td>
<td>(81)%</td>
<td>(81)%</td>
<td>(61)%</td>
<td></td>
</tr>
<tr>
<td>Small cell carcinomas (10)</td>
<td></td>
<td>1/10</td>
<td>0</td>
<td>0</td>
<td>1/10</td>
<td>1/10</td>
</tr>
<tr>
<td>(10)%</td>
<td></td>
<td>(10)%</td>
<td></td>
<td></td>
<td>(10)%</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers tested.  
* Percentage of positive tumors.

Francisco, CA). Dilutions of MAb ascites (COL-4, 1/2000; COL-12, 1/1000; B72.3, 1/1000; CC-49, 1/1000; CC-83, 1/1500) were established by end point titration on frozen and on paraffin sections. These concentrations were those which allowed maximal reactivity and absence of background. The same dilutions of MAb ascites were used for the preparation of anti-CEA, anti-TAG-72, and anti-(TAG-72 plus CEA) mixtures. Primary MAbs, diluted in RPMI 1640, were incubated for 30 min at room temperature (200 μl/slide). MAb B72.3 was also tested at 4°C in a humidified chamber for 12 h as suggested elsewhere (16). The peroxidase reaction was initiated by the addition of 0.06% diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in PBS containing 0.01% fresh hydrogen peroxide. After incubation for 7 min at room temperature, the slides were counterstained with Harris hematoxylin and were permanently mounted under a coverslip. These staining conditions were rigidly controlled to minimize variations in the intensity of the immunoreactivity in different experiments. The percentages of reactive cells were independently evaluated by two pathologists by counting the number of diaminobenzidine tetrahydrochloride stained cells out of a minimum of 200 carcinoma cells in 5 different areas defined by quadrant, using an oil immersion Apochromat objective at ×1000. In each case the same areas were identified and evaluated.

Fig. 2. Patterns of immunostaining observed with anti-TAG-72 and anti-CEA MAbs in lung carcinomas: cell membrane and cytoplasmic reactivity in a squamous cell carcinoma (MAb B72.3; A), predominant immunoreactivity of cytoplasmic vesicles in epinuclear position (arrow) in an adenocarcinoma (MAb COL-12; B). Immunoperoxidase, counterstained with hematoxylin, × 400.
on serial sections for their immunoreactivities with the different MAbs. The percentages of positive cells were reproducible and were consistent with the overall immunoreactivities of neoplastic cells in the tumor section, as evaluated by microscopic observation at low magnification. The reactivity was defined as focal when a percentage of positive cells could not be defined because the immunostaining was restricted to few neoplastic cells localized in a specific area of the tumor section. Mean percentages of antigen-positive cells, used to facilitate comparisons of the reactivities of MAbs or of their mixtures, were the means of the percentages of immunostained cells derived from all antigen-positive tumors of a given histotype. For this purpose cases with focal reactivity, which contained a number of positive cells far below 1%, were scored with a value of 0%. Negative controls were performed by replacing both the anti-CEA and anti-TAG-72 MAbs with a murine monoclonal IgG1 or IgG2a directed to an antigen not expressed in human cells, and with PBS.

RESULTS

To evaluate potential losses of immunoreactivity due to fixation, we compared formalin-fixed, paraffin-embedded sections with frozen sections obtained from the same tumor samples. In the 10 cases from which frozen tissues were available, the immunoreactivities of the different antibodies tested were comparable in paraffin-embedded and in frozen sections. However, paraffin sections were superior in morphological detail.

The reactivity of the anti-CEA and anti-TAG-72 MAbs with normal pulmonary tissues was limited to a focal immunostaining of apical cell membranes (anti-CEA) and of epinuclear vesicles in the Golgi area (anti-TAG-72) in bronchial epithelium and in some mucinous bronchial glands (Fig. 1). Type I and type II pneumocytes, alveolar septa, and intraalveolar macrophages did not show any immunostaining with the MAbs tested.

Initially, the study consisted in the evaluation of the reactivity of each MAb with the three major histological subsets of lung tumors, i.e., adenocarcinomas, squamous cell carcinomas, and small cell carcinomas. The anti-CEA MAbs COL-4 and COL-12 and the anti-TAG-72 MAbs B72.3, CC-49, and CC-83 yielded consistent immunohistochemical reactions with adenocarcinomas and squamous cell carcinomas, whereas small cell carcinomas were negative or, in only a few cases, were focally positive (Table 1). Several immunostaining patterns of carcinoma cells were common to the anti-CEA and the anti-TAG-72 MAbs. In squamous cell carcinomas and in poorly differentiated adenocarcinomas the immunostaining was localized either in the cytoplasm or along the cell membranes (Fig. 2A). Adenocarcinomas with tubular differentiation demonstrated enhanced reactivity of the apical cell membranes and secretion products or of perinuclear vesicles in the Golgi area (Fig. 2B).

In TAG-72 and/or CEA-positive cases the reactivity of secretion products was similar with the different MAbs.

The number of cases reactive with each antibody and the percentage of positive cells in each tumor reflected the heterogeneous expression of the specific CEA and TAG-72 epitopes in adenocarcinomas and squamous cell carcinomas (Table 1; Fig. 3). Adenocarcinomas were generally more reactive than squamous cell carcinomas with all the anti-CEA and anti-TAG-72 MAbs; this was apparent both in terms of number of positive cases and in percentage of positive cells (Table 1; Fig. 3). The numbers of adenocarcinomas reactive with each MAb were fairly similar, ranging from 84% (21 of 25) for CC-83 to 96% (24 of 25) for B72.3 and CC-49 (Table 1). However, there were differences in terms of number of immunostained cells. In this respect, CC-49 tended to react with higher percentages of adenocarcinoma cells than all the other antibodies under study (Fig. 34). In squamous cell carcinomas there was a decrease in the expression of CEA and TAG-72, which was particularly marked for the epitopes defined by MAbs CC-83 and COL-12 (Table 1). In fact, the percentages of tumors positive with anti-TAG-72 MAbs decreased to 81% (29 of 36) for CC-49 and B72.3 and to 61% (22 of 36) for CC-83. Similarly, CEA-positive tumors decreased to 75% (27 of 36) for COL-4 and 50% (18 of 36) for COL-12 (Table 1). The reduction in percentages of immunostained cells was even more remarkable (Fig. 3B). As previously observed in adenocarcinomas, squamous cell carcinomas more frequently expressed the TAG-72 epitopes defined by MAbs B72.3 and CC-49. However, in squamous cell carcinomas, B72.3 and not CC-49 was the antibody which tended to react with higher percentages of tumor cells (Fig. 3B).

The variability of epitope expression at the level of individual tumors, even within the same histological type, is a major caveat to the selection of a specific MAb on the basis of its ability to react with the highest number of tumors and with the highest average percentage of tumor cells. This was apparent when we analyzed the immunohistochemical reactivities of the anti-CEA and anti-TAG-72 MAbs on an individual basis. Fig. 4 illustrates the expression of the different CEA and TAG-72 epitopes in selected individual cases of adenocarcinoma and squamous cell carcinoma. The adenocarcinoma from patient 3 was negative with COL-12 (anti-CEA) and with CC-83 (anti-TAG-72) but was diffusely positive with COL-4 (anti-CEA), CC-49, and B72.3 (anti-TAG-72). In contrast, the adenocarcinoma from patient 16 contained very few cells reacting with COL-4 and B72.3, but was highly reactive with COL-12, CC-49, and CC-83. The adenocarcinoma from patient 24 gave heterogeneous reactions with the three anti-TAG-72 MAbs and was homogeneously positive with COL-12, but contained only few cells...
Fig. 8). All the adenocarcinomas reacted with the three mixtures (Table 2). Moreover, the immunodetection of 100% of carcinoma cells was observed in 7 cases with the anti-CEA mixture, in 8 cases with the anti-TAG-72 mixture, and in 13 cases with the anti-(TAG-72 plus CEA) mixture (Fig. 8/4). As previously shown, the immunodetection of 100% of carcinoma cells was obtained in 6 adenocarcinomas by using the single MAb with the highest reactivity (CC-49; Fig. 3A). The increase in the percentages of immunoreactive cells was therefore particularly significant with the anti-(TAG-72 plus CEA) mixture (Figs. 3A

reacting with COL-4. Squamous cell carcinomas from patients 2 and 3 were diffusely positive with B72.3, whereas their reactivities with all the other MAbs were restricted to much lower percentages of cells. However, the tendency to high levels of expression of the B72.3 epitope in squamous cell carcinomas was not apparent in the tumor from patient 33. Photomicrographs documenting dramatic differences in the immunostaining obtained in serial sections of the same tumors with MAbs directed to distinct epitopes of the same antigen are illustrated in Figs. 5-7.

The differential and noncoordinate expression of epitopes of the same antigenic molecule, which was apparent from the previous observations, suggested that mixtures of MAbs could increase the number of immunoreactive tumors and of detectable carcinoma cells within a given tumor, as compared to the number of cases and of tumor cells which could be detected by each individual MAb. To verify this hypothesis we utilized mixtures of (a) MAbs COL-4 and COL-12 (anti-CEA); (b) MAbs B72.3, CC-49, and CC-83 (anti-TAG-72); and (c) anti-(TAG-72 plus CEA). The use of mixtures resulted in an increase in the number of positive tumors and, particularly in adenocarcinomas, in the percentages of immunoreactive cells (Table 2;
and 8.A). In squamous cell carcinomas only anti-TAG-72 and anti-(TAG-72 plus CEA) mixtures resulted in an increase in the number of positive cases (Table 2; Fig. 8B). In fact the anti-CEA mixture did not increase the number of antigen-positive squamous carcinomas as compared to the results obtained with COL-4 alone (Tables 1 and 2; Figs. 3B and 8B). If we consider the number of squamous carcinomas which contained 100% of immunoreactive cells, it is possible to observe that the anti-(TAG-72 plus CEA) mixture gave an increase of only one case relative to the result obtained with the best single MAb (B72.3) and with the anti-TAG-72 mixture (5 versus 4 cases). The comparison of the mean percentages of cellular reactivity obtained with single MAbs and with mixtures revealed no significant differences in squamous cell carcinomas, whereas a cumulative staining effect was apparent in adenocarcinomas (Figs. 3 and 8; compare Fig. 3A to 8A and 3B to 8B). Individual examples of cumulative immunostaining are shown in adenocarcinomas 8 and 9 (Fig. 9).

DISCUSSION

In this report, we used 2 MAbs against CEA and 3 MAbs against TAG-72, all directed to distinct epitopes (20–23), to study the heterogeneity of antigen and/or epitope expression in a panel of 71 lung carcinomas, including 25 adenocarcinomas, 36 squamous cell carcinomas, and 10 small cell carcinomas. The specific CEA and TAG-72 epitopes were significantly expressed only in carcinomas of non-small cell type, which was
Table 2 Immunoperoxidase staining of lung tumors with anti-CEA, anti-TAG-72, and anti-(TAG-72 plus CEA) mixtures

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>Anti-CEA</th>
<th>Anti-TAG-72</th>
<th>Anti-(TAG-72 + CEA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(100)*</td>
<td>(100)*</td>
<td>(100)*</td>
</tr>
<tr>
<td>Squamous cell carcinomas</td>
<td>27/36</td>
<td>33/36</td>
<td>34/36</td>
</tr>
<tr>
<td></td>
<td>(75)*</td>
<td>(91)*</td>
<td>(96)*</td>
</tr>
</tbody>
</table>

* Numbers tested.

| Percentage of positive tumors. |

Fig. 7. Same area in serial sections of the squamous cell carcinoma 17 showing heterogeneous expression of the TAG-72 epitopes (A, CC-49; B, B72.3; C, CC-83).

consistent with results previously obtained with MAb B72.3 (16) and with several studies which discriminated between the two subsets of lung carcinomas on the basis of the antigenic phenotype (25–31). All the TAG-72 and CEA epitopes studied were expressed more in adenocarcinomas than in squamous cell carcinomas, both in terms of number of positive cases and in terms of percentages of immunoreactive cells. There were no major differences in the numbers of adenocarcinomas reacting with all these antibodies, but it should be noted that MAb CC-49 tended to decorate higher percentages of cells. In squamous cell carcinomas the CEA and TAG-72 epitopes, respectively, defined by MAb COL-12 and CC-83, were expressed at distinctly lower levels. With regard to the percentages of immunostained squamous carcinoma cells, MAb B72.3 tended to be superior. However, there were significant quantitative differences in the expression of distinct epitopes of the same antigenic molecule in several tumors, indicating that not only CEA and TAG-72, but also each epitope of the two antigens, could be expressed independently. In fact, these marked individual differences suggested a variability in the antigenic structure of the TAA molecules synthetized by lung carcinomas. The influence of fixation may not be responsible for the differential immu-
the increase in immunostaining obtained with the mixtures of MABs was much less significant and appeared mainly related to anti-TAG-72 reactivity.

ACKNOWLEDGMENTS

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ANTI-CEA AND ANTI-TAG-72 MAbs IN LUNG CARCINOMAS


Complementary Reactivities of Anti-Carcinoembryonic Antigen and Antitumor-associated Glycoprotein 72 Monoclonal Antibodies in Lung Carcinomas


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